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Freezing Tolerance of Bulb Scales of Lily Cultivars: Effects of Freezing and Storage Duration and Partial Dehydration

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Summary

Effects of freezing duration, previous storage duration of bulbs at −2 °C, and partial dehydration of scales on freezing tolerance of lily (*Lilium* hybrids) scales were studied for a series of cultivars. Freezing tolerance of scales was estimated by measuring ion leakage and recording scale bulblet regeneration. Both methods gave similar results. Freezing tolerance decreased with freezing duration. A longer previous storage duration of the bulbs at −2 °C tended to reduce freezing tolerance of the scales. Dehydration of the scales to 10–20 % loss of water content significantly increased freezing tolerance. Further dehydration to 30–40 % loss of water content did not further increase freezing tolerance. Nucleation temperatures, temperatures during crystallisation and melting temperatures of the scales were recorded for the cultivar ‹Enchantment›. Nucleation occurred at higher temperatures after a longer previous storage duration of bulbs, indicating a reduced capacity to remain supercooled. The increased freezing tolerance of dehydrated lily scales could partly be explained by a decreased melting temperature of the scales. We conclude long term storage of lily bulbs at −2 °C to be safer after partial dehydration to 10–20 % loss of the original water content.

Key words: Lilium, bulb, dehydration, freezing tolerance, frost, germplasm, ice formation, ion leakage, LT50, storage, thermocouple.

Abbreviations: FTcon = freezing tolerance estimated by conductivity of external solution; LT50 = the temperature that is lethal to 50 % of the tested material; TT50 = the temperature at which 50 % of the scales had thawed.

Introduction

In order to maintain a vegetative lily germplasm collection, bulbs can be stored for two to three years in moist peat at −2 °C (Bonnier et al., 1996). Increasing the maximum length of the storage period would make the maintenance of a lily collection more efficient. Although a temperature of −2 °C is generally used to store lily bulbs, sometimes injury of sprouts occurs (Beattie and White, 1993).

Freezing tolerance can be increased by cold-acclimatization, by abscisic acid, partial dehydration, or low atmospheric pressure (Halloy and Gonzales, 1993; Lång et al., 1994; Robertson et al., 1994; Mantyla et al., 1995). For storing a genetically diverse vegetative germplasm collection it is important to know the variation in freezing tolerance among genotypes. For long term storage, it is also important to know whether freezing tolerance is constant during storage. If freezing tolerance declines with storage duration, freezing injury can occur during prolonged storage.

Differences in freezing tolerance between plants can be based on differences in capacity to tolerate the formation of extracellular ice, on differences in osmotic potential of plant tissues and on differences in capacity to retain a supercooled state during freezing (Sutcliffe, 1977; Lipp et al., 1994). The formation of intracellular ice is lethal to the cells (Sutcliffe, 1977; Levitt, 1980), unless special freezing techniques are used for cryopreservation (Withers, 1991).

The freezing of a cell suspension is described by Steponkus (1984). During cooling both the cells and the suspending medium initially supercool. Subsequently ice nucleation occurs in the suspending medium at a temperature dependent on the freezing point of the medium and the presence of ice-nucleating agents. Ice formation will continue until the chemical potential of water in the unfrozen portion is in equilibrium with that of the ice. The intracellular solution must also come into equilibrium with the extracellular ice. Equilibrium is achieved either by intracellular ice formation or cell dehydration, dependent on the cooling rate and the stability of the plasma membrane.

The aim of our experiments was to determine freezing tolerance of different lily genotypes, and the way this tolerance is influenced by freezing duration, previous storage duration of bulbs, and partial dehydration of scales. Nucleation temperature, maximum temperature during freezing and melting temperature of scales were determined, with the aim to link freezing tolerance of lily scales to supercooling capacity, osmotic potential, or capacity to tolerate ice formation.

Material and Methods

Lily (*Lilium* hybrids) bulbs of ‹Avignon›, ‹Enchantment›, ‹Gelria›, ‹Mont Blanc› and ‹Star Gazer› with a circumference between 12 and 16 cm were obtained in autumn of 1992 and 1993 from commercial stocks. The bulbs were stored in moist peat at −2 °C. At the start of each experiment, bulbs were defrosted at 5 °C for three days. Then, the scales were removed and white scales were selected for uniformity. Three experiments were performed. In the *first experiment,* eight bulbs per genotype were used of ‹Avignon›, ‹Gelria›, ‹Mont Blanc› and ‹Star Gazer›, stored for 0.7 years. Freezing tolerance was determined by exposing three scales per genotype to −2, −4, −5, −6, and −8 °C for 24 h and by exposing six scales per genotype to −2, −3, −4, −5, and −6 °C for 144 h in a cooled incubator (Sanyo MIR-552, fluctuation ± 1 °C). In the *second experiment* freezing tolerance of ‹Gelria› scales from bulbs stored for 0.3 and 1.3 years (25 bulbs each) was estimated. Half of the scales from the bulbs stored for 0.3 years were air-dried at 17 °C under ventilation, until the loss of water content was between 10 and 20 %. The original water content of the scales (after storage, before drying) was 1.73 ± 0.16 g g^{-1} dry weight. The other scales were stored at 0 °C, until freezing tolerance was measured. Six scales per treatment were exposed to −2, −4, −6, −8, and −10 °C for 24 h to determine freezing tolerance. In the *third experiment* freezing tolerance of ‹Enchantment› scales from bulbs stored for 0.5, 1.5 and 3.5 years (50 bulbs each) at −2 °C was estimated. Scales were air-dried at 17 °C under ventilation into three classes of water content loss: 0−5 %, 10−20 %, and 30−40 %. The original water content of the scales was 1.42 ± 0.08, 1.75 ± 0.24 and 1.80 ± 0.14 g g^{-1} dry weight for scales from 0.5, 1.5 and 3.5 years stored bulbs respectively. Six scales per treatment were exposed to −2, −4, −6, −8, −10, and −20 °C for 144 h to determine freezing tolerance. Also, three ‹Enchantment› scales per treatment were used to measure the temperatures in the scales at the start of crystallisation (nucleation temperature), during crystallisation and during melting, using a copper/constantan thermocouple and a recorder.

Determination of freezing tolerance

After exposure to freezing temperatures scales were defrosted at 20 °C for 1 h and ion leakage tests were performed as described earlier (Bonnier et al., 1994). Each scale was rinsed in distilled water and placed in 100 mL of distilled water at 20 °C. After 1.5 h, the conductivity of the external medium was measured using a digital conductivity meter (Philips PW9526 with electrode PW9514/60). The leakage from each scale was corrected for the conductivity of the distilled water (1.85 µS cm^{-1}) and the initial fresh weight (before drying) of the scale. Freezing tolerance (FTcon) was estimated from the inflection point of a Gompertz-curve fitted by non-linear regression of log transformed electrical conductivity values on freezing temperatures. A Gompertz-curve is a sigmoidal curve, where the start of the increase is more abrupt than the end of the increase (Payne et al., 1993). After measuring ion leakage, the scales were planted in moistened vermiculite and incubated at 25 °C for 8 weeks. Then, the percentage of the scales that had formed scale bulblets was determined as a measure of survival. Survival of the scales was regressed on freezing temperature using a generalized linear model for proportions (Payne et al., 1993). Freezing tolerance was determined by the LT50, the temperature that is lethal to 50 % of the tested material (Dallaire et al., 1994; Maier et al., 1994; Mantyla et al., 1995). The fitting of scale survival (Fig. 1 A) and ion leakage (Fig. 1 B) after exposure to freezing is presented for the three dehydration classes of scales of 0.5 years stored ‹Enchantment› bulbs. The fitting of the curves for the other treatments was similar (results not shown).

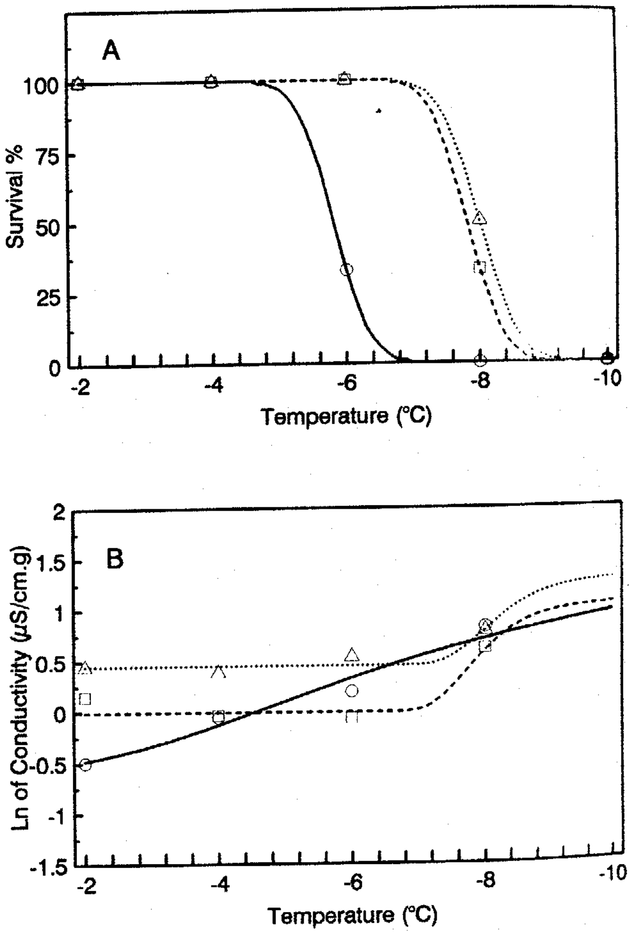

Fig. 1: The fitting of scale survival **(A)** and ion leakage **(B)** on freezing temperature of lily scales ‹Enchantment› after 6 days exposure to freezing. Before freezing, scales were air-dried and distributed in three classes of water content loss: 0−5 % (——O——), 10−20 % (- - - -□- - - -), and 30−40 % (· · · · △ · · · ·). Scales were taken from bulbs stored for 0.5 years in moist peat at −2 °C. Survival was determined by the percentage of scales that regenerated scale bulblets after freezing, and fitted by a generalized linear model for proportions. Ion leakage was measured by the electrical conductivity of external solution after 1.5 h leakage of lily scales and fitted by a Gompertz curve. Each point represents the mean of 6 scales.

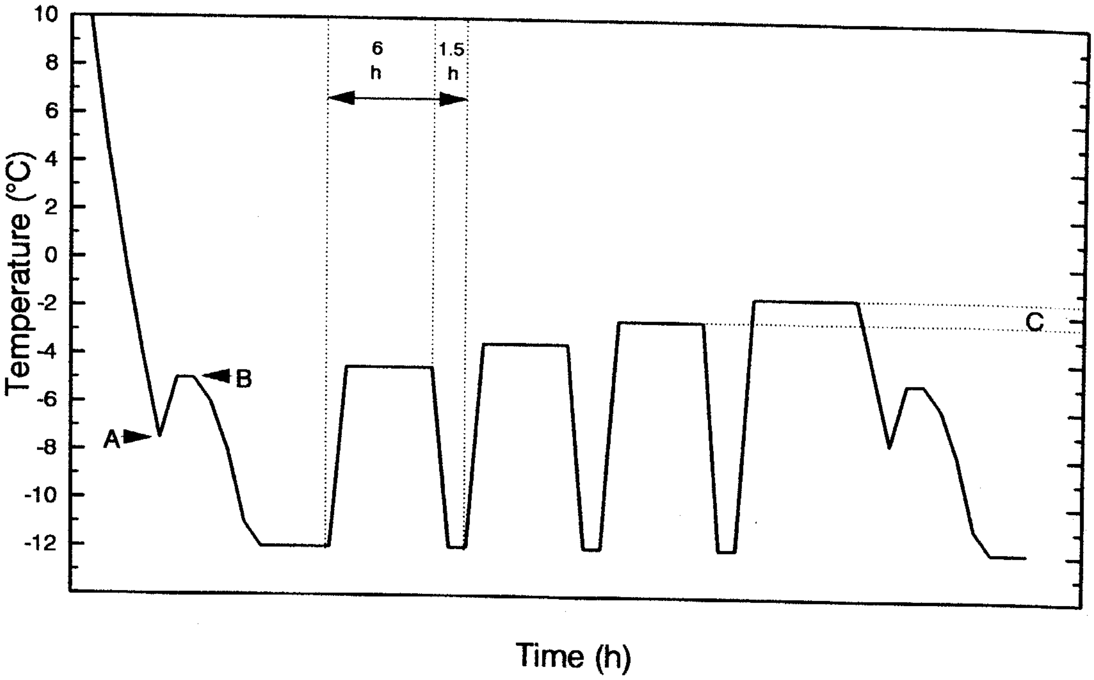

Fig. 2: Determination of the nucleation temperature (**A**), the maximum temperature during freezing (**B**), and the melting temperature range (C) of lily scales. The temperature was regulated by a cooled incubator and was measured in the scales by a copper/constantan thermocouple. Further method description: see Material and Methods.

Crystallisation in the scales

Scales were placed in the cooled incubator and the temperature was decreased from 10 °C to –12 °C in approximately 30 min. The nucleation temperature and the temperature during crystallisation were determined per scale as described by Levitt (1980). The start of ice crystallisation was observed by a sudden increase in temperature, rising from the nucleation temperature to the maximum temperature during freezing during crystallisation (Fig. 2). An accurate estimate of melting temperatures during a constant rise of temperature was not possible, because a clear halt of the temperature rise inside the scales during the melting was not observed. The melting temperature was estimated by increasing the temperature of frozen scales stepwise with 1 °C every 6 h, starting at –4.5 °C. After each temperature step, a 1.5 h temperature decrease to –12 °C was given. If scales had thawed, an exotherm was observed during this cooling (Fig. 2). The proportion of thawed scales per temperature was regressed on the temperature using a generalized linear model for proportions, giving estimates for the temperature at which half of the scales had thawed (TT50). Student t-tests were used to calculate significances ($P \leq 0.05$) for differences between storage duration of bulbs and dehydration classes as to the nucleation temperature and the maximum temperature during freezing. Significances ($P \leq 0.05$) between melting temperatures (TT50's) were calculated by using the rank sum test of Mann-Whitney for two independent samples (Snedecor and Cochran, 1980).

Results

Determination of freezing tolerance

Scales exposed to 24 h freezing survived lower temperatures than those exposed to 144 h freezing, the difference ranging from 0.7 °C (‹Mont Blanc›) to 2.0 °C (‹Avignon›) for bulbs previously stored for 0.7 year (Table 1). This means that freezing tolerance decreased with freezing duration. Longer duration than 144 h will probably further decrease freezing tolerance. Freezing tolerance was also genotype dependent. ‹Mont Blanc› was relatively tolerant to freezing, whereas ‹Gelria› was more sensitive (Table 1). The LT50 of non-dehydrated scales from 0.7 year stored bulbs, varied between –4.9 and –7.0 °C after 24 h freezing and between –4.1 and –6.3 after 144 h freezing (Table 1).

Freezing tolerance of non-dehydrated scales tended to decrease with longer previous storage duration of bulbs, but the results were not significant. The LT50 after 24 h freezing exposure of non-dehydrated ‹Gelria› scales stored for 1.3 years was higher than the LT50 of 0.3-years-stored and 0.7-years-stored ‹Gelria› scales (Table 1). In addition, the LT50 after 144 h of freezing exposure of non-dehydrated scales from 1.5 years stored bulbs of ‹Enchantment› was higher than that of non-dehydrated scales of 0.5 years stored ‹Enchantment› bulbs (Table 1). Unfortunately, freezing tolerance of 3.5 years stored ‹Enchantment› bulbs could not be determined, because survival was less than 100 %, and ion leakage was increased of scales that were not exposed to freezing. For dehydrated scales, no indication was found for a decrease in freezing tolerance with previous storage duration of bulbs.

Dehydration of scales increased freezing tolerance significantly for all tested bulbs. LT50 of scales from 0.3-years-stored ‹Gelria› bulbs decreased 1.2 °C after 10–20 % loss of water content and LT50 of scales from 0.5 and 1.5-years-stored ‹Enchantment› bulbs decreased 2.0 and 1.5 °C, respec-

Table 1: Freezing tolerance (LT50 + 95 % confidence interval) of scales from lily bulbs stored for different times at −2 °C, dehydrated and distributed in three classes of water content loss, exposed for 24 h and 144 h to freezing. The initial water content of the scales from 0.3 and 1.3 years stored Gelria bulbs was $1.73 \pm 0.16\,g\,g^{-1}$ dry weight. The initial water content of the scales from 0.5, 1.5 and 3.5 years stored ‹Enchantment› bulbs was 1.42 ± 0.08, 1.75 ± 0.24 and $1.80 \pm 0.14\,g\,g^{-1}$ dry weight respectively. The initial water content of other scales was not determined.

Cultivar	Storage duration (year)	Year of harvest	% loss of initial water content	Estimated LT50 (°C)	95 % confidence interval
Freezing exposure = 24 h					
Gelria	1.3	1992	0–5	−4.2	(−3.4;−5.1)
Gelria	0.7	1992	0–5	−4.9	(−4.4;−5.4)
Gelria	0.3	1993	0–5	−5.8	(−4.9;−6.6)
Gelria	0.3	1993	10–20	−7.0	(−6.4;−7.6)
Avignon	0.7	1992	0–5	−6.1	(−5.6;−6.8)
Star Gazer	0.7	1992	0–5	−6.1	(−5.6;−6.8)
Mont Blanc	0.7	1992	0–5	−7.0	(−6.4;−7.6)
Freezing exposure = 144 h					
Gelria	0.7	1992	0–5	−4.1	(−3.7;−4.6)
Avignon	0.7	1992	0–5	−4.1	(−3.7;−4.6)
Star Gazer	0.7	1992	0–5	−5.1	(−4.7;−5.4)
Mont Blanc	0.7	1992	0–5	−6.3	(−6.0;−7.1)
Enchantment	1.5	1993	0–5	−5.3	(−4.8;−5.8)
Enchantment	0.5	1993	0–5	−5.8	(−4.9;−6.5)
Enchantment	0.5	1993	10–20	−7.8	(−7.0;−8.5)
Enchantment	0.5	1993	30–40	−8.0	(−7.1;−8.9)
Enchantment	1.5	1992	10–20	−8.4	(−8.1;−9.2)
Enchantment	1.5	1992	30–40	−8.4	(−8.1;−9.2)

tively, after 10–20 % loss of water content (Table 1). Dehydration of ‹Enchantment› scales to 30–40 % loss of water content did not further increase freezing tolerance (Table 1). For dehydrated scales, there was no indication of a decrease in freezing tolerance with previous storage duration of bulbs. Dehydration of scales caused increased ion leakage at −2 °C, without influence on survival (Fig. 1 A, B).

Freezing tolerance estimated by survival of the scales (LT50) and freezing tolerance estimated by ion leakage of the scales (FTcon) gave similar results (FTcon = 0.97 * LT50 − 0.16) and were highly correlated ($r^2 = 0.90$).

Crystallisation in the scales

The nucleation temperature was higher in scales from 3.5-years-stored ‹Enchantment› bulbs than in scales from 0.5- and 1.5-years-stored bulbs, but duration of previous storage had no influence on the maximum temperature during freezing and the melting temperature of the scales (Fig. 3 A). These results indicate a reduced capacity of scales from 3.5-years-stored bulbs to remain supercooled.

Dehydration of scales did not have a significant effect on the nucleation temperature (Fig. 3 B). The maximum temperature during freezing was not significantly changed after 10–20 % loss of the water content, but dehydration to 30–40 % loss of the original water content significantly decreased this temperature. Dehydration of scales decreased their melting temperatures significantly (Fig. 3 B).

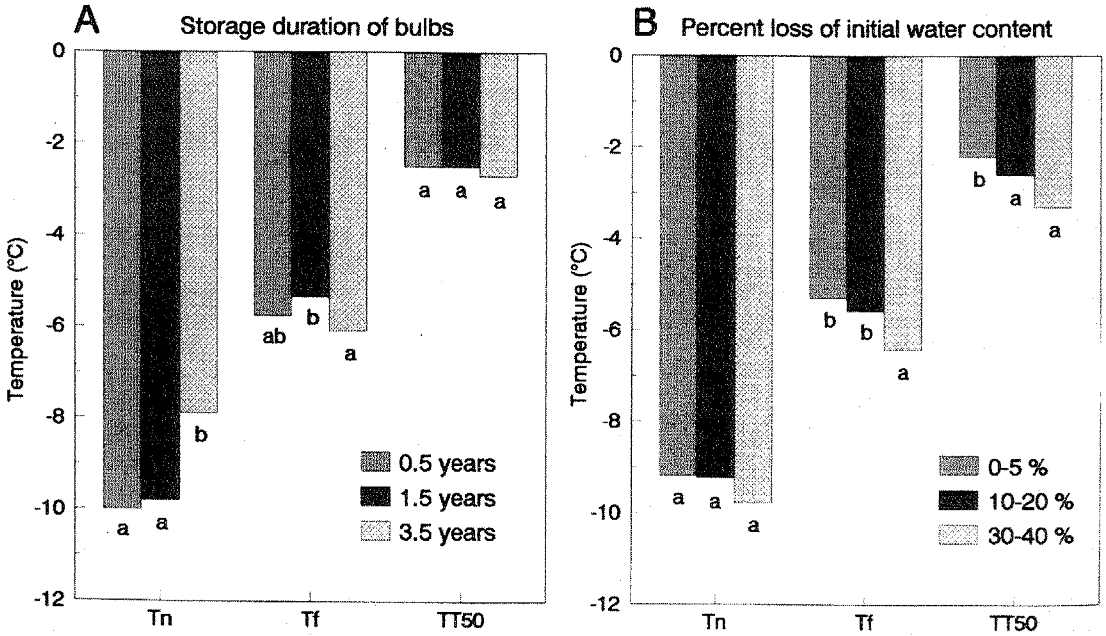

Fig. 3: Means of nucleation temperature (Tn), means of maximum temperature during freezing (Tf), and estimates of melting temperature (TT50) of lily scales ‹Enchantment› exposed to a temperature declining from 10 °C to −12 °C in about 30 minutes. **(A)**: Scales were taken from bulbs stored previously for 0.5, 1.5 and 3.5 years at −2 °C; (B): Scales were air-dried and distributed into three classes of water content loss (all the three storage durations). Different minor characters indicate significant differences ($P \leq 0.05$) between treatments. Nine to twelve scales were used per treatment.

Discussion

Significant differences in freezing tolerance were found between cultivars. The cultivars with the lowest freezing tolerance determine the minimum storage temperature of a germplasm collection. These cultivars were ‹Avignon› and ‹Gelria›, which both had an LT50 of −4.1 °C after 144 h freezing exposure. However, freezing damage occurs probably at higher temperatures during prolonged storage, because freezing tolerance declined with freezing duration, and injury of sprouts is sometimes observed during prolonged storage of lily bulbs at −2 °C (Beattie and White, 1993).

Freezing tolerance of lily scales tended to decline with previous storage duration of bulbs at −2 °C, but this effect was not significant. Storage of bulbs increased the nucleation temperature and had no effect on the maximum temperature during freezing and the melting temperature of the scales. The difference between nucleation temperature and equilibrium freezing temperature can be used as a criterion for supercooling capacity (Lipp et al., 1994). The maximum temperature during freezing is lower or equal to the equilibrium freezing temperature, otherwise, the freezing process would stop. The melting temperature is the upper limit of the equilibrium freezing temperature. Therefore, the results indicate a reduced capacity of scales to remain supercooled. Retaining a state of supercooling during freezing is one of the ways plants can avoid freezing damage by ice crystallisation (Lipp et al., 1994). Supercooling was demonstrated for all scales by the difference in nucleation temperature and the maximum temperature during freezing.

Dehydration to 10−20 % loss of water content increased freezing tolerance of ‹Gelria› and ‹Enchantment›. These results are in accordance with earlier reports on other plants (Lång et al., 1994; Pearson and Davison, 1994; Silim and Lavender, 1994; Mantyla et al., 1995). Further dehydration to 30-40 % loss of water content did not significantly further increase freezing tolerance.

The plasmalemma is a primary site of freezing injury leading to cell leakage (Steponkus, 1984; Samygin, 1994). Therefore, ion leakage can be used to determine freezing injury (Bonnier et al., 1992; Bigras and Calme, 1994; Maier et al., 1994). Freezing tolerance can be estimated by the inflection point of a sigmoidal curve fitted by non-linear regression of ion leakage on temperature (Fry et al., 1991; Maier et al., 1994). We estimated freezing tolerance (FTcon) by the inflection point of a Gompertz-curve fitted by non-linear regression (Payne et al., 1993) of the natural logarithm of electrical conductivity of external solution on temperature. FTcon and LT50 were nearly equal and highly correlated. FTcon was measured 8 wks earlier than LT50.

Freezing injury in plants is a complex phenomenon that has been extensively studied (Levitt, 1956, 1980; Samygin, 1994; Steponkus, 1984; Sutcliffe, 1977). Freezing of tissue results in the formation of extracellular or intracellular ice, dependent on cooling rate and the presence of ice-nucleating agents. At low cooling rates, ice formation is extracellular and water efflux from cells concentrates the intracellular solutes, decreasing the intracellular freezing point (Steponkus, 1984). The formation of extracellular ice can lead to injury, but not necessarily. The injury is dependent on plant species, plant tissue, cooling rate, length of freezing period, and thawing conditions (Levitt, 1980). At high cooling rates, water efflux from cells is not sufficiently rapid and intracellular ice formation may follow supercooling of the intracellular solution (Steponkus, 1984). In our experiment the cooling rate was 44 °C per h. We could not distinguish between extracellular or intracellular ice formation in the lily scales.

Though freezing tolerance of 10−20 % dehydrated ‹Enchantment› scales was increased by 2.0 °C, significant changes in nucleation temperature were not found, and maximum temperature during freezing was only decreased significantly after 30−40 % dehydration (Fig. 3 B). The melting temperature of 10−20 % dehydrated ‹Enchantment› scales was only slightly decreased. This means, that the increase in freezing tolerance is not fully explained by the decrease in melting temperature.

During storage at a supercooled state, crystallisation can start at any moment. Crystallisation is probably lethal to the scales and scales seem to lose their capacity to retain a supercooled state during long term storage. Therefore, lily bulbs should not be stored for a prolonged period at a supercooled state. We conclude, that the risk of freezing injury of lily scales during prolonged storage at −2 °C can be decreased by dehydrating the scales to 10−20 % loss of the original water content.

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